ANDROLOGY

ISSN: 2047-2919

ANDROLOGY

ORIGINAL ARTICLE

Correspondence:
Rui Manuel Reis, Molecular Oncology Research Center, Barretos Cancer Hospital, 1331, Rua Antenor Duarte Villela St, CEP 14784 400, Barretos, São Paulo, Brazil.
E-mail: ruireis.hcb@gmail.com

Keywords:
microsatellite instability, mutation, proto-oncogene protein B-raf, testicular germ cell tumors, testicular neoplasms

Received: 4-Sep-2015
Revised: 16-Mar-2016
Accepted: 18-Mar-2016

doi: 10.1111/andr.12200

Absence of microsatellite instability and *BRAF* (*V600E*) mutation in testicular germ cell tumors

[1,2]F. M. Cárcano, [3,4]A. H. Lengert, [3,4]D. O. Vidal, [3,5]C. Scapulatempo Neto, [6]L. Queiroz, [6]H. Marques, [7,8]F. Baltazar, [3]G. N. Berardinelli, [3]C. M. S. Martinelli, [5]E. C. A. da Silva, [3,7,8]R. M. Reis and [3,4]L. F. Lopes

[1]Department of Medical Oncology, Barretos Cancer Hospital, [2]Barretos School of Health Sciences, Dr. Paulo Prata – FACISB, [3]Molecular Oncology Research Center, Barretos Cancer Hospital, [4]Barretos Children's Cancer Hospital, [5]Department of Pathology, Barretos Cancer Hospital, Barretos, Brazil, [6]Department of Medical Oncology, Hospital de Braga, [7]Life and Health Sciences Research Institute (ICVS), Health Sciences School, University of Minho, and [8]ICVS/3B's-PT Government Associate Laboratory, Braga, Guimarães, Portugal

SUMMARY

Testicular germ cell tumors (TGCT) are the most common malignant neoplasm in young men. DNA mismatch repair deficiency can lead to microsatellite instability (MSI), an important mechanism of genetic instability. A mutation of the *BRAF* gene has been implicated in the pathogenesis of several solid tumors and has recently become an important therapeutic target. The role of MSI and *BRAF* gene mutation in TGCT, particularly in refractory disease, is poorly understood and reported findings are controversial. In this study, we aimed to determine the frequency and clinical impact of MSI status and *BRAF* mutations in TGCT. DNA was isolated from formalin-fixed paraffin embedded (FFPE) tissue from 150 TGCT cases. The MSI phenotype was evaluated using multiplex PCR for five quasimonomorphic mononucleotide repeat markers. Exon 15 of the *BRAF* oncogene (V600E) was analyzed by PCR, followed by direct sequencing. Sixteen percent of cases were considered to have refractory disease. In a small subset of cases (17 for MSI and 18 for *BRAF*), the quantity and quality of DNA recovery were poor and therefore, were unable to be analyzed. The remaining 133 TGCT cases showed a complete absence of MSI. Of the 132 cases successfully evaluated for *BRAF* mutations, all were V600E wild-type. In conclusion, despite a distinct response of testicular germ cell tumors to therapy, microsatellite instability, and the *BRAF* V600E mutation were absent in all testicular germ cell tumors tested in this study.

INTRODUCTION

Testicular germ cell tumors (TGCT) are the most frequent malignant neoplasm found in young men and represent 95% of testicular cancers (Bray *et al.*, 2006). Commonly, TGCTs are classified into two distinct groups, seminomas (SE) and non-seminomas (non-SE). The mainstay treatment for both over the last few decades has been platinum-based chemotherapy and surgery, leading to robust response and cure rates (Einhorn, 2002; Feldman, 2015). Patients with advanced disease achieve a 5-year-overall survival of more than 70% (Ries *et al.*, 2007), while those with non-SE have a poorer prognosis and 5-year-overall survival of 50% (IGCCCG, 1997). Fifteen percent of all patients will develop refractory disease representing a challenge in the clinical management (Mead *et al.*, 2005; Lorch *et al.*, 2010).

The integrity of the genome depends on the coordinated action of DNA repair genes, also known as 'caretakers' (Kinzler & Vogelstein, 1997). The failure of one type of such caretakers, a DNA mismatch repair gene, contributes to genome instability by making microsatellite regions more susceptible to mismatch, leading to what is termed microsatellite instability (MSI) (Imai & Yamamoto, 2008; Shah *et al.*, 2010). The MSI phenotype is a hallmark of hereditary non-polyposis colorectal cancer (HNPCC) syndrome, but is also present in 10–15% of sporadic colorectal cancers (Kinzler & Vogelstein, 1996; Hamelin *et al.*, 2008; Gryfe, 2009). MSI phenotype has also been described in other cancers including endometrial and gastric (Hamelin *et al.*, 2008). Recently, colorectal and non-colorectal tumors with identified mismatch-repair defects have been reported as more responsive to a new anti–programmed death 1 immune checkpoint inhibitor drug, highlighting a new role for MSI in immunotherapeutic prediction (Le *et al.*, 2015).

The *BRAF* oncogene is an important part of the MAPK (Mitogen Activated Protein Kinase) cellular signaling pathway and related to cell proliferation, differentiation and survival (Kolch,

2000). *BRAF* is activated by hotspot mutations, mainly V600E, which has been found in melanomas and other types of cancer (Rajagopalan *et al.*, 2002; Michaloglou *et al.*, 2008). Recently, the *BRAF* V600E mutation has emerged as a therapeutic target in melanoma, changing the natural history of the disease (Chapman *et al.*, 2011).

In TGCT, the roles of MSI and *BRAF* mutations are poorly characterized and controversial results have been reported (Huddart *et al.*, 1995; Mayer *et al.*, 2002; Velasco *et al.*, 2004, 2008; Sommerer *et al.*, 2005; Honecker *et al.*, 2009; Masque-Soler *et al.*, 2011). Therefore, in the present study, we evaluated the presence and clinical impact of MSI and *BRAF* mutations in a large series of TGCTs.

MATERIALS AND METHODS

Patients and specimens

Formalin-fixed paraffin-embedded (FFPE) tissues from 150 consecutive cases of testicular germ cell tumors were retrieved from the Biobank of the Departments of Pathology at Barretos Cancer Hospital (Brazil) and Hospital de Braga (Portugal). All patients were diagnosed between 2006 and 2012 and all analyzed samples were collected from primary tumors at diagnosis prior to the use of any systemic treatment. All TGCT cases were independently re-evaluated by a pathologist for diagnosis confirmation.

Germ cell tumor cell lines

The germ cell tumor cell lines NTERA-2, 1411H, 1777N and N2102Ep Clone2/A6 were purchased from the European Collection of Cell Cultures (ECACC). Cell lines were cultured in DMEM containing 2 mM glutamine and 10% FBS (Sigma Aldrich, St. Louis, MO, USA). After achieving 80% confluence, cell lines were trypsinized, washed twice with 1% PBS, and centrifuged. DNA was isolated using the TRIZOL reagent (Life Technologies, Bethesda, MD, USA) and manufacturer's recommendations were followed.

DNA isolation from FFPE tissue

DNA was obtained from FFPE tissue sections representative of tumor pathology as previously described (Martinho *et al.*, 2009a, b; Yamane *et al.*, 2014), with minor modifications. Briefly, 10 μm thick unstained sections of paraffin blocks were sectioned and one H&E section was used for identification and selection of the tumor area, which was then macrodissected into a microfuge tube using a sterile needle (Neolus, 25G – 0.5 mm). The macrodissected tissue was deparaffinized by a serial wash with xylol and ethanol (100–70–50%) and allowed to air-dry. DNA was isolated using the QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quality and concentration of DNA were measured using a Nanodrop 2000 Spectrophotometer and stored at −20 °C until molecular analysis.

Microsatellite instability analysis

Microsatellite instability evaluation was performed using multiplex PCR for five quasimonomorphic mononucleotide repeat markers (NR-27, NR-21, NR-24, BAT-25, and BAT-26) as previously described (Viana-Pereira *et al.*, 2011; Campanella *et al.*, 2014; Yamane *et al.*, 2014). Briefly, each antisense primer was end-labeled with a fluorescent dye: FAM (6-carboxyfluorescein) for BAT-26 and NR-21, VIC (2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein) for BAT-25 and NR-27, and NED (2,7,8-benzo-5-fluoro-2,4,7-trichloro-5-carboxyfluorescein) for NR-24. PCR was performed using the Qiagen Multiplex PCR Kit (Qiagen) with 0.5 μL of DNA at 50 ng/μL. All five markers were co-amplified in a standard multiplex PCR (denaturation at 95 °C for 15 min, 40 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 90 sec and extension at 72 °C for 30 sec, followed by a final extension at 72 °C for 40 min). PCR products were then submitted to capillary electrophoresis on an ABI 3500xL Genetic Analyzer (Applied Biosystems, Austin, TX, USA) according to the manufacturer's instructions. Results were analyzed using GENEMAPPER v4.1 (Applied Biosystems) software.

In a recent study, our group determined the quasimonomorphic variation range (QMVR) of each marker for the Brazilian population (Campanella *et al.*, 2014). Accordingly, samples were considered MSI-H when two or more markers were altered and MSI-low (MSI-L) when only one marker was altered, with further validation by MSI analysis of normal tissue or immunohistochemistry of the mismatch repair (MMR) enzymes. Samples were considered microsatellite stable (MSS) when none of the markers were altered.

The HCT-15 colorectal cancer cell line was used as a positive control of MSI-High (MSI-H) in all MSI analyses. DNA from HCT-15 cells was extracted using the TRIZOL reagent (Life Technologies) following the manufacturer's protocol.

In 10% of samples, the MSI analysis was repeated for quality control.

Mutational analysis of *BRAF*

Hotspot regions (exon 15) of the oncogene *BRAF* (codon 600) were analyzed by polymerase chain reaction (PCR), followed by direct sequencing, as previously described by our group (Basto *et al.*, 2005; Martinho *et al.*, 2009a,b; Yamane *et al.*, 2014).

Polymerase chain reactions were performed in a final volume of 15 μL, according the following conditions: 1.5 μL buffer (Qiagen); 2 mM $MgCl_2$ (Qiagen); 100 mM dNTPs (Invitrogen, Carlsbad, CA, USA); 0.3 mM of both sense and anti-sense primers (Sigma Aldrich), 1 unit of HotStarTaq DNA polymerase (Qiagen) and 1 μL of DNA. The *BRAF* primers used were: 5′-TCATAATGCTTGCTCTGATAGGA-3′ (sense) and 5′-GGCCAAAAATTTAATCAGTGGA-3′ (antisense) (Basto *et al.*, 2005; Martinho *et al.*, 2009a,b; Yamane *et al.*, 2014). The PCR was performed with a Veriti thermocycler (Applied Biosystems) and products were evaluated by electrophoresis in 1% agarose gel.

All PCR products were purified with EXO-SAP (GE Techonology, Cleveland, OH, USA), and submitted for a sequencing reaction using 1 μL of BigDye (Applied Biosystems), 1.5 μL of sequencing buffer (Applied Biosystems) and 1 μL of primer. Sequencing reactions were followed by post-sequencing purification with EDTA, alcohol and sodium citrate. PCR products were eluted in Hi-Di (formamide) and incubated at 95 °C for 5 min and subsequently cooled at −4 °C for at least 5 min. Direct sequencing was performed on an ABI 3500 series Genetic Analyzer (Applied Biosystems).

In 10% of samples, the *BRAF* mutation analysis was repeated for quality control.

Statistical analysis

We assessed measurements of frequency, central tendency, and dispersion for clinical and pathological characteristics. The overall survival was assessed using Kaplan–Meier methods. All diagnostics were performed by orchidectomy before any systemic therapy. Date of diagnosis was the starting point for the survival analysis and events were defined as all-cause mortality. Patients lost to follow-up or alive at the time of this analysis were censored. Survival curves were compared using Logrank tests. The two-sided *p*-values were considered statistically significant at <0.05. All statistical analyses were performed using SPSS 19.0 software (IBM Corp, Armonk, NY, USA).

RESULTS

One hundred and fifty cases of TGCT were retrieved. After DNA isolation, 17 cases of MSI and 18 cases of *BRAF* yielded inconclusive results as a result of poor quality and quantity of extracted DNA. The clinicopathological features of all validated cases are summarized in Table 1.

The mean age of diagnosis was 30 years old and the majority of cases (~68%) had non-SE. The predominant histologies identified were mixed tumor followed by seminoma. All stages of disease were observed, while advanced disease was the most frequent stage identified. Among those who received chemotherapy, around 16% were considered to have refractory disease after treatment with the first-line chemotherapy regimen, BEP (Bleomicine, Etoposide and Cisplatin). A majority of cases were classified as having intermediate or high-risk disease according to IGCCCG. Median follow-up was 36.0 months for cases evaluating MSI and 35.5 months for *BRAF* evaluated cases. Five-year overall survival was 84.5 and 83.2% for MSI and *BRAF* evaluated cases, respectively.

Table 1 Clinicopathological features of testicular germ cell tumor (TGCT) valid cases

	Patients	
Characteristics	*BRAF* *N* (%)	MSI *N* (%)
TGCT valid	132	133
Age (year)		
Mean (SD)	30 (9.9)	30 (10.0)
Range	1–63	1–63
Histological group		
Non-seminoma	90 (68.2)	91 (68.4)
Seminoma	42 (31.8)	42 (31.6)
Histology		
Mixed tumor	53 (40.2)	54 (40.6)
Seminoma	42 (31.8)	42 (31.6)
Embryonal carcinoma	16 (12.1)	17 (12.8)
Yolk Sac tumor	10 (7.6)	9 (6.8)
Immature teratoma	6 (4.5)	6 (4.5)
Mature teratoma	3 (2.3)	3 (2.3)
Choriocarcinoma	2 (1.5)	2 (1.5)
Serum tumor markers (AJCC)		
S0	18 (13.6)	17 (12.8)
S1	31 (23.5)	30 (22.6)
S2	38 (28.8)	37 (27.8)
S3	23 (17.4)	25 (18.8)
SX	22 (16.7)	24 (18)
Staging (AJCC)		
I	28 (21.2)	28 (21.1)
IS	20 (15.2)	21 (15.8)
II	25 (18.9)	26 (19.5)
III	59 (44.7)	58 (43.6)
Chemosensitivity		
Responsive	84 (63.6)	84 (63.2)
Refractory	20 (15.2)	21 (15.8)
No chemotherapy	28 (21.2)	28 (21.1)
IGCCCG risk		
Good	31 (23.5)	31 (23.3)
Intermediate	17 (12.9)	17 (12.8)
Poor	27 (20.5)	27 (20.3)
Not applicable	48 (36.4)	49 (36.8)
Missing	9 (6.8)	9 (6.8)

SD: Standard deviation; AJCC: American Joint Committee on Cancer; IGCCCG: International Germ Cell Cancer Cooperative Group.

Among treatment-refractory patients, the mean age was 29 (min: 20 years and max: 51 years) and 87% of tumors were non-SE. A single case was categorized as stage II, while all other cases were stage III. Eighty-five percent of valid cases presented with two or more sites of metastasis and 95% were classified as high or intermediate risk according IGCCCG (data not shown).

The overall survival of responsive and refractory patients to chemotherapy in the cases evaluated for MSI or *BRAF* mutation are demonstrated in Fig. 1A and B, respectively. Refractory cases had a poorer overall survival rate compared to responsive cases.

We obtained results of MSI status for 88.7% (133/150) of patients. 126 had a microsatellite stable (MSS) genotype, 7 had MSI-L and none had MSI-H. In a previous study (Campanella *et al.*, 2014), our group reported that in order to accurately determine MSI-L, the MSI markers of tumor tissue should be compared with the germ-line tissue of the patient. Therefore, for the seven cases with MSI-L, we isolated adjacent normal tissue and identified the presence of an identical MSI marker profile, thereby indicating a MSS phenotype.

The mutation analysis of *BRAF* V600E was successful in 88% (132/150) of TGTC cases. None were determined to have a *BRAF* V600E mutation.

All germ cell tumor cell lines were observed to have MSS and wild type *BRAF*.

DISCUSSION

In our study, we demonstrated an absence of MSI in all TGCT analyzed even in the refractory cases and cell lines. These results are in accordance with several studies, which also did not observe such alterations in TGCT (Lothe *et al.*, 1995; Faulkner & Friedlander, 2000; Olasz *et al.*, 2005; Vladusic *et al.*, 2014) (Table 2). In contrast, another study reported the presence of MSI at a significant frequency (33%) and as a potential biomarker of refractory TGCT and poor clinical outcome (Mayer *et al.*, 2011). Several factors may explain this discrepancy, including differences in methodology, tumor subtype, and ethnically distinct populations. Indeed, the data are still controversial, with MSI frequencies varying from 0 to 33%, and assessing a limited number of cases (Table 2).

Distinct methodologies have been used in the assessment of MSI in TGCT (Table 2), including the use of specific microsatellite markers from mononucleotides to tetranucleotides, each of which could lead to different results, as reported for EMAST, an MSI form associated with tetranucleotide repeats (Devaraj *et al.*, 2010; Carethers *et al.*, 2015). Herein, we used a validated methodology that comprised five quasimonomorphic mononucleotide repeat markers to identify MSI (NR-27, NR-21, NR-24, BAT-25, and BAT-26) as previously described (Viana-Pereira *et al.*, 2011; Yamane *et al.*, 2014; Campanella *et al.*, 2015). BAT-

Figure 1 Overall survival of refractory and responsive testicular germ cell tumors cases. (A) microsatellite instability sample; (B) *BRAF* mutation sample.

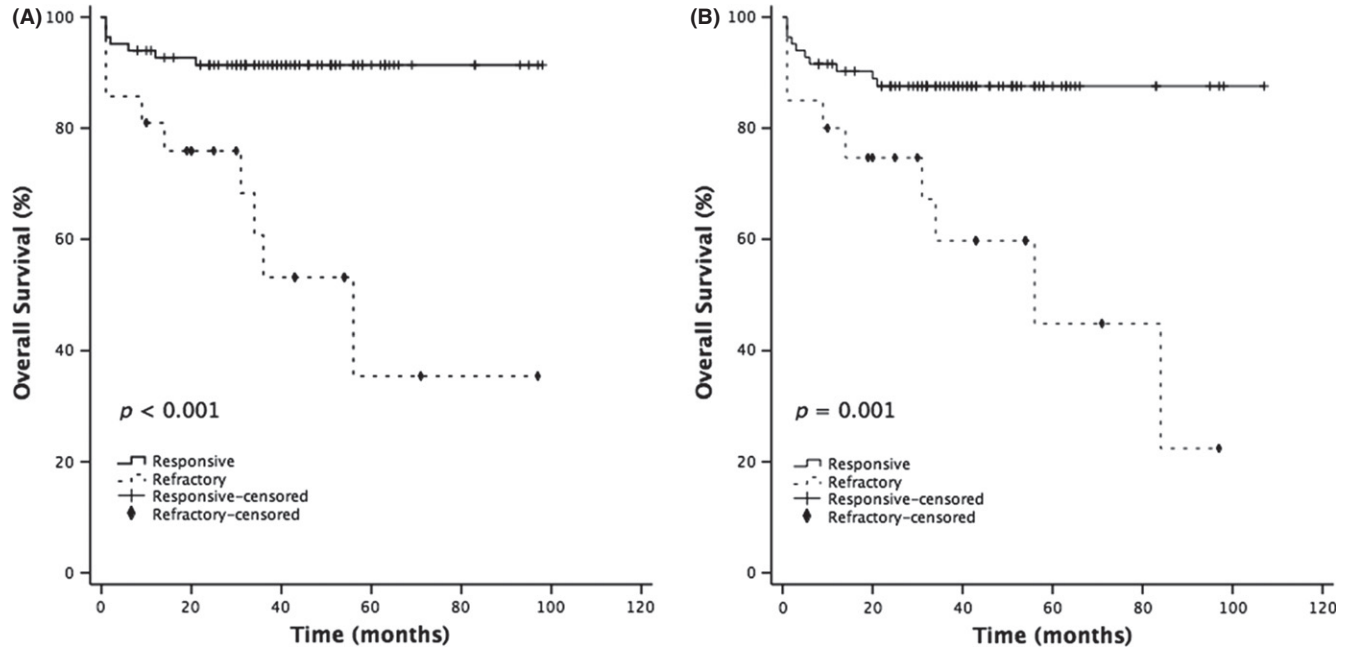

Table 2 Summary of studies assessing MSI in testicular germ cell tumors (TGCT)

Author, year	Sample (*N*) Total/Refractory	Age (years)	Country	MSI high (%) Total/Refractory	MSI markers
Huddart *et al.* (1995)	29/NR	NR	United Kingdom	21/NA	Dinucleotide (D1S216,D2S123, D16S303, D17S796) and tetra- and tri-nucleotide repeats.
Lothe *et al.* (1995)	31/NR	NR	Norway, Finland and Denmark	0/NA	32 microsatellite loci (dinucleotide repeats)
Faulkner & Friedlander (2000)	24/NR	NR	Australia	0/NA	78 microsatellite loci (di- and tetra-nucleotide repeats)
Velasco *et al.* (2004)	118/NR	16–45	Chile	25/NA	Mononucleotide (BAT25, BAT26) and dinucleotide (D2S123, D3S1029, D3S1283, D3S1293, D9S66, D9S113, LNSCA and TP53CA)
Sommerer *et al.* (2005)	62/0	NR	Germany	6/NA	Mononucleotide (BAT25, BAT26, BAT40) and dinucleotide (D2S123, D5S346, MSH6)
Olasz *et al.* (2005)	51/15	17–60	Hungary	0/0	Mononucleotide (BAT25, BAT26) and dinucleotide (D2S123, D5S346, D17S250)
Honecker *et al.* (2009)	135/35	14–66	Germany and Netherlands	7/26	Mononucletide (BAT25, BAT26, BATRII, BAT40) and dinucleotide (D2S123, D5S346, D17S250, MSH6)
Mayer *et al.* (2011)[a]	12/NR	17–48	Germany and Netherlands	33/NA	Mononucleotide (BAT25, BAT26) and dinucleotide (D2S123, D5S346, D17S250)
Vladusic *et al.* (2014)	40/NR	17–60	Croatia	0/NA	BAT-26 (mononucleaotides) and 8 microsatellite loci (dinucleotide repeats)
Current study	133/20	1–63	Brazil and Portugal	0/0	Mononucleotide (NR-27, NR-21, NR-24, BAT-25, BAT-26)

NR: not reported; NA: not applicable. [a]Late relapses.

25, BAT-26 (mononucleotides) and D5S346, D2S123, D17S250 (dinucleotides) were classically recommended by Bethesda panel for MSI evaluation until 2002 (Boland *et al.*, 1998), when an international consensus updated the recommendation and recommended that dinucleotide repeats be substituted for mononucleotide repeats because of improved sensitivity (Buhard *et al.*, 2004). We utilized this new recommended methodology for this study – a methodology that has been recently validated by our group in the Brazilian population (Campanella *et al.*, 2014).

Other potential causes of differing results may be associated with tumor aggressiveness. Mayer *et al.* (2002) assessed 111 TGCT cases and 11 of these were refractory to treatment. The authors found a higher rate of MSI in the refractory cases compared to treatment-responsive cases (45 vs. 6%, $p = 0.001$). It is noteworthy that among refractory cases, the MSI group achieved better median progression free-survival (26 months vs. 6 months, $p = 0.05$). Although, it is not clear how TGCT was treated in both groups. One hundred unselected cases from study by Mayer *et al.* were used later as a control cohort in the study by Honecker *et al.* (2009) (Table 2). Velasco *et al.* (2008) assessed the MMR enzyme expression and MSI in 162 TGCT patients, with a majority having seminomas. The authors found a negative correlation between MSI and survival. However, the rate of advanced and high-risk disease was unknown, which may lead to a misinterpretation of the results.

Table 3 Summary of studies assessing *BRAF* mutation in testicular germ cell tumors (TGCT)

Author, year	Sample (*N*) Total/ Refractory	Age (range)	Country	*BRAF* mutation (%) Total/ Refractory
McIntyre *et al.* (2005)	65/NR	NR	United Kingdom	0/NA
Sommerer *et al.* (2005)	62/0	NR	Germany	5/NA
Honecker *et al.* (2009)	135/35	14–66	Germany and Netherlands	7/26
Piulats *et al.* (2010)	75/25	16–56	Spain	0/0
Mayer *et al.* (2011)[a]	12/NR	17–48	Germany and Netherlands	28/NA
Masque-Soler *et al.* (2011)	66/15	1–20	Germany	0/0
Satpute *et al.* (2013)	59/33	NR	United States	0/0
Feldman *et al.* (2014)	70/46	14–60	United States	0/0
Current study	132/20	1–63	Brazil and Portugal	0/0

NR: not reported; NA: not applicable. [a]Late relapses.

Despite these data, Olasz *et al.* (2005) studied 15 cases of treatment refractory and 36 treatment-responsive TGCT cases and although they found MSI in 31.4% of patients, none of cases was MSI-high and there was no correlation with any clinical variable, including resistance to treatment. Interestingly, Piulats *et al.* (2009) did not identify the MSI in a nude mice model with xenografts of germ cell tumor refractory to cisplatin.

Another biomarker analyzed in the present study was the *BRAF* V600E mutation. Herein, we did not identify any case or cell line harboring a *BRAF* mutation, in accordance with the majority of the studies that addressed this issue after McIntyre *et al.* (2005) (Table 3). Nevertheless, some authors have reported *BRAF* mutations in TGCT, with a frequency up to 28% (Table 3). Interestingly, Honecker *et al.* (2009) found that resistant tumors had a higher incidence of *BRAF* V600E mutations compared with unselected tumors (26 vs. 1%, $p = 0.0001$) and, for the first time, a correlation between *BRAF* V600E and cisplatin resistance was reported. Nevertheless, Piulats *et al.* also assessed *BRAF* mutation status in 75 men with germ cell tumors; 84% of cases were non-SE and one-third of all cases were refractory to cisplatin. None of these cases exhibited a BRAF V600E mutation (Piulats *et al.*, 2010). More recently, Satpute *et al.* (2013) analyzed 59 germ-cell tumors, among these more than half were treatment-refractory, and no *BRAF* mutations were identified. Additionally, Feldman *et al.* (2014) found no BRAF mutations in 46 GCT refractory cases, the largest number of refractory cases already reported. Interestingly, the TCAM2 seminoma cell line was initially reported to exhibit a *BRAF* mutation (de Jong *et al.*, 2008). However, other studies did not corroborate these findings even in other germ cell tumor cell lines (Goddard *et al.*, 2010; Feldman *et al.*, 2014).

Our study is a retrospective analysis and therefore has an inherent selection bias. However, our data are consistent with findings from other studies that show MSI and *BRAF* mutations are not present in TGCT. This series of 150 cases is very heterogeneous and representative of several types of germ cell tumor histologies, clinical staging, and response rates to chemotherapy. The predominance of advanced disease and non-SE histologies in our series differs from the classical literature, where seminomas and stage I disease are the most common (Ries *et al.*, 2007). Our hospital is a reference center for TGCT, and affiliated with the Brazilian Childhood Germ Cell Tumor Study Group, a consortium developed to standardize the diagnostic assessment and multidisciplinary treatment of TGCT patients in Brazil (Lopes *et al.*, 2009). For this reason, our series might be biased by more advanced cases. However, the several risk groups were well represented in the study.

In conclusion, contrasting to other solid tumors, microsatellite instability and *BRAF* V600E mutation are not present in testicular germ cell tumors, even in treatment-refractory cases. It is necessary to target other pathways and explore other aspects of genetics and epigenetics in TGCT to better understand its biology and identify new theranostic biomarkers.

ACKNOWLEDGEMENTS

This project was financially supported by Barretos Cancer Hospital internal research funds (PAIP). The authors acknowledge Dr. Laura Musselwhite for her critical review of the manuscript.

AUTHORS' CONTRIBUTION

RMR and FMC designed the study. FMC, LQ and HM contributed to data recovery and management. CSN, ECAS and FB retrieved the biologic samples and prepared them for the genetic analysis. The genetic analysis was performed by FMC, AHL, and CMSM, under supervision of DOV and RMR. The cell lines were evaluated by AHL, under supervision of DOV and RMR. GNB, AHL, DOV, and RMR participated in MSI analysis and mutational analysis of *BRAF*. FMC, DOV, LFL, and RMR drafted the manuscript, analyzed the data, and critically reviewed and discussed the results. All authors read and accepted the final manuscript.

COMPETING INTERESTS

The authors have no competing interests.

ETHICS APPROVAL

The study was conducted following national and institutional ethical policies, and it was previously approved by the Barretos Cancer Hospital Ethical Committee (protocol CAAE 12297713.0.0000.5437). The ethical committee classified this study as having minimal risk, ensuring confidentiality, and not resulting in any clinical influences because changes in treatment or genetic counselling for the participants and their families. For these reasons, the ethical committee waived the need for consent.

REFERENCES

Basto D, Trovisco V, Lopes JM, Martins A, Pardal F, Soares P & Reis RM. (2005) Mutation analysis of B-RAF gene in human gliomas. *Acta Neuropathol* 109, 207–210.

Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN & Srivastava S. (1998) A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res* 58, 5248–5257.

Bray F, Ferlay J, Devesa SS, McGlynn KA & Moller H. (2006) Interpreting the international trends in testicular seminoma and nonseminoma incidence. *Nat Clin Pract Urol* 3, 532–543.

Buhard O, Suraweera N, Lectard A, Duval A & Hamelin R. (2004) Quasimonomorphic mononucleotide repeats for high-level microsatellite instability analysis. *Dis Markers* 20, 251–257.

Campanella NC, Berardinelli GN, Scapulatempo-Neto C, Viana D, Palmero EI, Pereira R & Reis RM. (2014) Optimization of a pentaplex panel for MSI analysis without control DNA in a Brazilian population: correlation with ancestry markers. *Eur J Hum Genet* 22, 875–880.

Campanella NC, Penna V, Ribeiro G, Abrahao-Machado LF, Scapulatempo-Neto C & Reis RM. (2015) Absence of microsatellite instability in soft tissue sarcomas. *Pathobiology* 82, 36–42.

Carethers JM, Koi M & Tseng-Rogenski SS. (2015) EMAST is a form of microsatellite instability that is initiated by inflammation and modulates colorectal cancer progression. *Genes (Basel)* 6, 185–205.

Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, Jouary T, Schadendorf D, Ribas A, O'Day SJ, Sosman JA, Kirkwood JM, Eggermont AM, Dreno B, Nolop K, Li J, Nelson B, Hou J, Lee RJ, Flaherty KT, McArthur GA; BRIM-3 Study Group. (2011) Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med* 364, 2507–2516.

Devaraj B, Lee A, Cabrera BL, Miyai K, Luo L, Ramamoorthy S, Keku T, Sandler RS, McGuire KL & Carethers JM. (2010) Relationship of EMAST and microsatellite instability among patients with rectal cancer. *J Gastrointest Surg* 14, 1521–1528.

Einhorn LH. (2002) Curing metastatic testicular cancer. *Proc Natl Acad Sci USA* 99, 4592–4595.

Faulkner SW & Friedlander ML. (2000) Microsatellite instability in germ cell tumors of the testis and ovary. *Gynecol Oncol* 79, 38–43.

Feldman DR. (2015) Update in germ cell tumours. *Curr Opin Oncol* 27, 177–184.

Feldman DR, Iyer G, Van Alstine L, Patil S, Al-Ahmadie H, Reuter VE, Bosl GJ, Chaganti RS & Solit DB. (2014) Presence of somatic mutations within PIK3CA, AKT, RAS, and FGFR3 but not BRAF in cisplatin-resistant germ cell tumors. *Clin Cancer Res* 20, 3712–3720.

Goddard NC, McIntyre A, Gilbert D, Kitazawa S & Shipley J. (2010) No evidence for V600E BRAF mutation in the seminoma cell line TCam-2. *Genes Chromosom Cancer* 49, 963–966.

Gryfe R. (2009) Inherited colorectal cancer syndromes. *Clin Colon Rectal Surg* 22, 198–208.

Hamelin R, Chalastanis A, Colas C, El Bchiri J, Mercier D, Schreurs AS, Simon V, Svrcek M, Zaanan A, Borie C, Buhard O, Capel E, Zouali H, Praz F, Muleris M, Flejou JF & Duval A. (2008) Clinical and molecular consequences of microsatellite instability in human cancers. *Bull Cancer* 95, 121–132.

Honecker F, Wermann H, Mayer F, Gillis AJ, Stoop H, van Gurp RJ, Oechsle K, Steyerberg E, Hartmann JT, Dinjens WN, Oosterhuis JW, Bokemeyer C & Looijenga LH. (2009) Microsatellite instability, mismatch repair deficiency, and BRAF mutation in treatment-resistant germ cell tumors. *J Clin Oncol* 27, 2129–2136.

Huddart RA, Wooster R, Horwich A & Cooper CS. (1995) Microsatellite instability in human testicular germ cell tumours. *Br J Cancer* 72, 642–645.

IGCCCG. (1997) International Germ Cell Consensus Classification: a prognostic factor-based staging system for metastatic germ cell cancers. International Germ Cell Cancer Collaborative Group. *J Clin Oncol* 15, 594–603.

Imai K & Yamamoto H. (2008) Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics. *Carcinogenesis* 29, 673–680.

de Jong J, Stoop H, Gillis AJ, Hersmus R, van Gurp RJ, van de Geijn GJ, van Drunen E, Beverloo HB, Schneider DT, Sherlock JK, Baeten J, Kitazawa S, van Zoelen EJ, van Roozendaal K, Oosterhuis JW & Looijenga LH. (2008) Further characterization of the first seminoma cell line TCam-2. *Genes Chromosom Cancer* 47, 185–196.

Kinzler KW & Vogelstein B. (1996) Lessons from hereditary colorectal cancer. *Cell* 87, 159–170.

Kinzler KW & Vogelstein B. (1997) Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature* 386, 761–763

Kolch W. (2000) Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem J* 351(Pt 2), 289–305.

Le DT, Uram JN, Wang H, Bartlett BR, Kemberling H, Eyring AD, *et al.* (2015) PD-1 blockade in tumors with mismatch-repair deficiency. *N Engl J Med* 372, 2509–2520.

Lopes LF, Macedo CR, Pontes EM, Dos Santos Aguiar S, Mastellaro MJ, Melaragno R, Vianna SM, Lopes PA, Mendonca N, de Assis Almeida MT, Sonaglio V, Ribeiro KB, Santana VM, Schneider DT & de Camargo B. (2009) Cisplatin and etoposide in childhood germ cell tumor: brazilian pediatric oncology society protocol GCT-91. *J Clin Oncol* 27, 1297–1303.

Lorch A, Beyer J, Bascoul-Mollevi C, Kramar A, Einhorn LH, Necchi A, Massard C, De Giorgi U, Flechon A, Margolin KA, Lotz JP, Germa Lluch JR, Powles T & Kollmannsberger CK. (2010) Prognostic factors in patients with metastatic germ cell tumors who experienced treatment failure with cisplatin-based first-line chemotherapy. *J Clin Oncol* 28, 4906–4911.

Lothe RA, Peltomaki P, Tommerup N, Fossa SD, Stenwig AE, Borresen AL & Nesland JM. (1995) Molecular genetic changes in human male germ cell tumors. *Lab Invest* 73, 606–614.

Martinho O, Gouveia A, Viana-Pereira M, Silva P, Pimenta A, Reis RM & Lopes JM. (2009a) Low frequency of MAP kinase pathway alterations in KIT and PDGFRA wild-type GISTs. *Histopathology* 55, 53–62.

Martinho O, Longatto-Filho A, Lambros MB, Martins A, Pinheiro C, Silva A, Pardal F, Amorim J, Mackay A, Milanezi F, Tamber N, Fenwick K, Ashworth A, Reis-Filho JS, Lopes JM & Reis RM. (2009b) Expression, mutation and copy number analysis of platelet-derived growth factor receptor A (PDGFRA) and its ligand PDGFA in gliomas. *Br J Cancer* 101, 973–982.

Masque-Soler N, Szczepanowski M, Leuschner I, Vokuhl C, Haag J, Calaminus G & Klapper W. (2011) Absence of BRAF mutation in pediatric and adolescent germ cell tumors indicate biological differences to adult tumors. *Pediatr Blood Cancer* 59, 732–735.

Mayer F, Gillis AJ, Dinjens W, Oosterhuis JW, Bokemeyer C & Looijenga LH. (2002) Microsatellite instability of germ cell tumors is associated with resistance to systemic treatment. *Cancer Res* 62, 2758–2760.

Mayer F, Wermann H, Albers P, Stoop H, Gillis AJ, Hartmann JT, Bokemeyer CC, Oosterhuis JW, Looijenga LH & Honecker F. (2011) Histopathological and molecular features of late relapses in non-seminomas. *BJU Int* 107, 936–943.

McIntyre A, Summersgill B, Spendlove HE, Huddart R, Houlston R & Shipley J. (2005) Activating mutations and/or expression levels of tyrosine kinase receptors GRB7, RAS, and BRAF in testicular germ cell tumors. *Neoplasia* 7, 1047–1052.

Mead GM, Cullen MH, Huddart R, Harper P, Rustin GJ, Cook PA, Stenning SP & Mason M. (2005) A phase II trial of TIP (paclitaxel, ifosfamide and cisplatin) given as second-line (post-BEP) salvage chemotherapy for patients with metastatic germ cell cancer: a medical research council trial. *Br J Cancer* 93, 178–184.

Michaloglou C, Vredeveld LC, Mooi WJ & Peeper DS. (2008) BRAF(E600) in benign and malignant human tumours. *Oncogene* 27, 877–895.

Olasz J, Mandoky L, Geczi L, Bodrogi I, Csuka O & Bak M. (2005) Influence of hMLH1 methylation, mismatch repair deficiency and microsatellite instability on chemoresistance of testicular germ-cell tumors. *Anticancer Res* 25, 4319–4324.

Piulats JM, Nadal M, Martinez-Iniesta M, Puertas S, Gonzalez S, Vidal A, Condom E, Germa-Lluch J, Garcia Del Muro X & Villanueva A. (2009) Nude mice model of primary human

nonseminoma germ cell tumors to study biology and resistance to cisplatin treatment. In: *ASCO Annual Meeting Proceedings*, Vol. 27, p. e16143.

Piulats JM, Vidal A, Villanueva A, Munoz C, Pisa A, Nadal M, Germa-Lluch J, Condom E & Garcia del Muro X. (2010) MLH1 and BRAF status analysis in metastatic germ cell tumors. In: *ASCO Annual Meeting Proceedings*, Vol. 28, p. e15092.

Rajagopalan H, Bardelli A, Lengauer C, Kinzler KW, Vogelstein B & Velculescu VE. (2002) Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. *Nature* 418, 934.

Ries L, Young JL, Keel GE, Eisner MP, Lin YD & Horner M-J. (eds) (2007) SEER Survival Monograph: Cancer Survival Among Adults: U.S. SEER Program, 1988–2001, Patient and Tumor Characteristics. National Cancer Institute, SEER Program, NIH Pub. No. 07-6215, Bethesda, MD, 2007, 165–170.

Satpute SR, Koster R, Nathanson KL, Vaughn DJ, Albany C, Einhorn LH & Hanna NH. (2013) Retrospective, correlative study of BRAF mutation V600E in testicular cancer patients. In: *Journal of Clinical Oncology*, Vol. 31. Amer Soc Clinical Oncology 2318 Mill Road, STE 800, Alexandria, VA 22314, USA.

Shah SN, Hile SE & Eckert KA. (2010) Defective mismatch repair, microsatellite mutation bias, and variability in clinical cancer phenotypes. *Cancer Res* 70, 431–435.

Sommerer F, Hengge UR, Markwarth A, Vomschloss S, Stolzenburg JU, Wittekind C & Tannapfel A. (2005) Mutations of BRAF and RAS are rare events in germ cell tumours. *Int J Cancer* 113, 329–335.

Velasco A, Riquelme E, Schultz M, Wistuba II, Villarroel L, Koh MS & Leach FS (2004) Microsatellite instability and loss of heterozygosity have distinct prognostic value for testicular germ cell tumor recurrence. *Cancer Biol Ther* 3, 1152–1158; discussion 1159-1161.

Velasco A, Corvalan A, Wistuba II, Riquelme E, Chuaqui R, Majerson A & Leach FS. (2008) Mismatch repair expression in testicular cancer predicts recurrence and survival. *Int J Cancer* 122, 1774–1777.

Viana-Pereira M, Lee A, Popov S, Bax DA, Al-Sarraj S, Bridges LR, Stavale JN, Hargrave D, Jones C & Reis RM. (2011) Microsatellite instability in pediatric high grade glioma is associated with genomic profile and differential target gene inactivation. *PLoS ONE* 6, e20588.

Vladusic T, Hrascan R, Kruslin B, Pecina-Slaus N, Perica K, Bicanic A, Vrhovac I, Gamulin M & Franekic J. (2014) Histological groups of human postpubertal testicular germ cell tumours harbour different genetic alterations. *Anticancer Res* 34, 4005–4012.

Yamane LS, Scapulatempo-Neto C, Alvarenga L, Oliveira CZ, Berardinelli GN, Almodova E, Cunha TR, Fava G, Colaiacovo W, Melani A, Fregnani JH, Reis RM & Guimaraes DP. (2014) KRAS and BRAF mutations and MSI status in precursor lesions of colorectal cancer detected by colonoscopy. *Oncol Rep* 32, 1419–1426.

